

Apoptosis in epithelial cells of apical radicular cysts

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Abstract

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Aim To investigate the occurrence of apoptotic cell death in the epithelium of radicular cysts and to compare its frequency in lesions presenting a distinct functional state.

Methodology Twenty radicular cysts were selected and arranged into two groups with 10 lesions in each group: atrophic (quiescent) and hyperplastic (active) epithelium. Morphologic investigations of apoptosis were conducted by means of optic microscopy in haematoxylin and eosin slides. Immunohistochemical techniques to detect the bcl-2 protein were carried out by streptavidin–biotin–peroxidase assay. In both instances, 30 sequential high-power microscopic fields were observed to determine apoptotic (AI) and bcl-2

immunostaining (bcl-2I) indexes. The presence of AI and bcl-2I within the two groups was compared using the *t*-test. Correlation between the AI and the bcl-2I was investigated using the Spearman test.

Results Apoptosis was detected in the epithelium of all cysts. Higher AI levels were found in lesions with an atrophic (0.17 ± 0.19) rather than a hyperplastic (0.10 ± 0.10) epithelium. The same was found for the bcl-2I levels (0.06 ± 0.03 vs. 0.04 ± 0.01 , respectively). However, these differences were not statistically significant. A positive and significant correlation was found between AI and bcl-2I.

Conclusions Apoptosis was always present in the epithelium of the lesions and was more frequent in lesions with atrophic (quiescent) epithelium.

Keywords: apoptosis, immunohistochemistry, pathogenesis, radicular cyst.

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Introduction

Apical radicular cysts (ARC) are the most common cystic lesions in the jaws (Shear 1985). They represent a periapical inflammatory disease evoked by infected and necrotic dental pulp (Torabinejad 1983, Shear 1985, Meghji *et al.* 1996). In such circumstances, bacterial toxins (e.g. LPS) and inflammatory mediators (e.g. IL-1 and PGE₂) continuously released in the vicinity elicit alveolar bone resorption and its substitu-

tion by granulation tissue (apical granuloma) (Summers & Papadimitriou 1975, Meghji *et al.* 1996, Kawashima & Staschenko 1999, Gervásio *et al.* 2002). Because epithelial rests of Malassez are common in this area, other mediators, such as IL-6 and EGF, released into the periapical tissues are likely to induce proliferation of these epithelial cells, which is the central histogenetic event in the development of ARC (Torabinejad 1983, Meghji *et al.* 1996, Gervásio *et al.* 2002, Nickolaychuck *et al.* 2002). Furthermore, central portions of the increasing epithelial proliferative islands undergo necrosis since the inner cells become too remote from nutrition and respiratory sources in the connective tissue. Once a central cavity is formed, the lesion is named an apical radicular cyst. Enlargement of the lesion occurs by a continuous release of bone resorptive and other growth factors from inflamed

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connective tissue or water liquid penetration of the lumen by osmotic pressure (Torabinejad 1983, Nickolaychuck *et al.* 2002).

The morphological aspects of epithelial tissue have been considered to reflect functional activity of the ARC (Cury *et al.* 1998). Lesions depicting a thin, regular and atrophic layer of stratified squamous epithelium of up to 10 cells thick, usually with mild to moderate inflammatory infiltrate in the connective tissue are considered quiescent cysts. Hyperplastic stratified squamous epithelial cells of irregular but increased thickness, often arranged into proliferating arcades, and with severe infiltration of inflammatory cells into the capsule, have been considered characteristic of active lesions (Cury *et al.* 1998, Moreira *et al.* 2000). In spite of the increasing knowledge on the pathogenesis of periapical disease, mechanisms underlying cavity formation in ARC have not been studied recently.

Cellular death can be initiated by physiologic or pathogenic signals. Two broad mechanism of cell death are known: necrosis and apoptosis (Searle *et al.* 1982, Blagosklonny 2003, Loro *et al.* 2003, Satchell *et al.* 2003). Necrosis is a massive tissue destruction elicited by environmental signals surpassing cellular adaptive response. Such an effect is mediated mainly by the release of pro-inflammatory products that aggravate tissue damage, amplifying the initial pathologic stimulus (Blagosklonny 2003, Loro *et al.* 2003). Apoptosis, on the other hand, is a complex and genetically programmed cell death mechanism that participates in the homeostatic control of cell population in a fashion opposed to mitosis (Blagosklonny 2003, Satchell *et al.* 2003). Cell survival in the tissues depends on continuous arrival of positive signals (e.g. regular supply of oxygen). The lack of such stimuli activates apoptotic pathways to decrease the cellular population (Chu *et al.* 2002, Blagosklonny 2003, Loro *et al.* 2003, Renton *et al.* 2003). Apoptosis occurs in individual or small groups of cells and is initiated by specific signals (e.g. hypoxia, increasing proliferative activity) which activate intracellular active pathways leading to mitochondrial, cytoskeletal and nuclear changes. The apoptotic cell is then fragmented and macrophages or other neighbour cells engulf it, without the release of inflammatory mediators (Blagosklonny 2003, Loro *et al.* 2003, Satchell *et al.* 2003).

The bcl-2 family is a group of closely related proteins that plays a major role in apoptosis regulation (Adams & Cory 1998, Blagosklonny 2003). This is constituted by inductive (e.g. bax) and inhibitory (e.g. bcl-2) apoptotic factors, and cell survival is warranted by

higher inhibitory apoptotic gene expression. Bcl-2 is a 26 KD protein resident in the mitochondrial membrane, endoplasmic reticulum and nuclear envelope. This works as a prototypic inhibitory apoptotic factor, avoiding the increase in the mitochondrial membrane permeability that can be observed in the initial stages of apoptosis (Adams & Cory 1998, Blagosklonny 2003, Satchell *et al.* 2003).

The occurrence of apoptosis in the controlling of epithelial cell population in ARC has not been studied recently (Takahashi *et al.* 1999). The aim of this study was to determine if apoptotic cell death in the epithelium of ARC exists and compare its frequency to lesions with distinct functional state.

Methods

Twenty samples of ARC were selected for study. All the cysts showed a complete epithelial lining in the histologic sections evaluated. They were further classified according to their functional state of the epithelium as hyperplastic (10) or atrophic (10) (Cury *et al.* 1998, Moreira *et al.* 2000).

The morphologic evaluation of apoptosis was determined in new 5 µm tissue sections stained using the conventional H & E method. Two investigators were calibrated by observation of characteristic cellular alterations of this phenomenon in lesions with abundant apoptotic figures (lymphoma and squamous cell carcinoma samples). The following parameters were considered as evaluative factors: nuclear shrinkage with chromatin condensation and hyperchromasia, cytoplasmic condensation and shrinkage depicting intense eosinophilia, and detachment from neighbour cells (Goldsworthy *et al.* 1995, Loro *et al.* 2003). Thirty sequential high power microscopic fields (HPF, 1000× magnification) were observed for counting of apoptotic (but not bodies) and nonapoptotic epithelial cells. The length of observation was determined after bootstrapping and jack-knife statistical procedures (Ayres *et al.* 2003). The bootstrap and jack-knife technique involves drawing repeated samples (with replacement) from the data distribution, which in turn is, formulated by calculating a value of the statistic for each sample. The bootstrap can be used to obtain confidence intervals and, standard errors and as in this study, determine the number of fields for analysis (Wasserman & Bockenholt 1989).

Briefly, an immunohistochemical staining of the bcl-2 antigen was performed with the monoclonal antibody '124' (Dako, Carpinteria, CA, USA), 1 : 75

diluted in 5 mmol L⁻¹ Tris-HCl buffer with 1% bovine serum albumin. It was carried out in 3 µm tissue sections plated on silane-coated slides that were dewaxed in xylene, rehydrated in decreasing series of ethanol, submitted to steamer antigen retrieval in 1 mmol L⁻¹ ethylenediaminetetraacetic acid (EDTA) buffer, pH 8.2, for 30 min, blocked for intrinsic enzymatic activity, and then incubated with the primary antibody for 18 ho at 4 °C. The reactions were amplified using the LSAB + system (Dako), revealed with peroxidase activity on diaminobenzidine chromogene substrate, counterstained with Mayer's haematoxylin, dehydrated in crescent series of ethanol, cleared in xylene, and finally mounted in permount. Thirty HPF were also examined for the counting of bcl-2 positive and negative cells, as described above.

Apoptotic (AI) and bcl-2 immunohistochemical staining (bcl-2I) indexes were determined as the ratio between apoptotic or bcl-2 positive cells and the total number of cells. Comparison of the mean AI between groups with hyperplastic and atrophic epithelium was performed using the *t*-test. Possible correlation between the AI and bcl-2I were investigated using the Spearman test. Statistical significance was set at 5%. Calculations were computed using BioEstat software (Ayres *et al.* 2003).

Results

Apoptotic cells and bodies were seen in the epithelium of all cysts (Fig. 1a). The mean AI was 0.134 ± 0.188 (SD). Apoptosis was more frequent in lesions with atrophic (AI mean = 0.170 ± 0.247) rather than hyperplastic epithelium (0.098 ± 0.103). In the same manner, higher bcl-2I was found in lesions with atrophic (0.06 ± 0.03) than hyperplastic (0.04 ± 0.01) epithelium. These differences were not statistically significant. The immunohistochemical detection of bcl-2 antigen was observed as a slight cytoplasmic brown staining in individual cells (Fig. 1b). The mean bcl-2I was 0.073 ± 0.027 . A positive and significant correlation was found between AI and bcl-2I ($R = 0.71$, $P < 0.05$).

Discussion

The present work demonstrates the occurrence of apoptotic cell death in the epithelium of ARC. This phenomenon was observed in all of the lesions studied. In this study, light microscopy was used for detection and quantification of apoptosis in H & E slides, as opposed to techniques such as TUNEL. Although

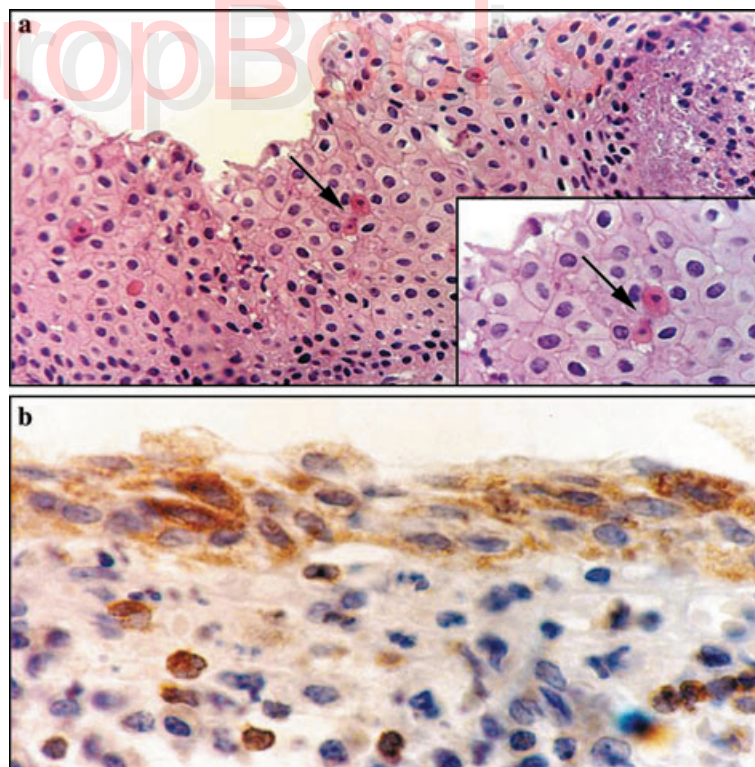


Figure 1 (a) Apoptotic cells in the epithelium of a radicular cyst, with characteristic cytoplasmic and nuclear condensation (H & E, 400×; insert = 1000×); (b) immunohistochemical staining of bcl-2 antigen in the epithelium of a radicular cyst, depicting characteristic brown colour (streptavidin-biotin-peroxidase, 400×).

supplemental techniques are sometimes helpful, a carefully studied H & E section gives a definitive assessment of apoptosis (Goldsworthy *et al.* 1995, Loro *et al.* 2003). With this method apoptosis has been detected with a high specificity, despite some loss of sensitivity. The same parameters were used to scan all samples, thereby strengthening the data.

Apoptosis in leukocytes in the connective tissue of the lesion has been shown, but no trial has been made to evaluate its role in epithelial cell clearance in ARC (Takahashi *et al.* 1999). In the endodontic literature, cavitation in epitheliated granulomas occurs when the inner cells of the proliferative epithelial islands become distant from the support connective tissue (Summers & Papadimitriou 1975, Torabinejad 1983). The resulting ischaemia decreases the ATP levels in the cell, breaking down electrolytic pumps, and allowing cytoplasmic Ca^{2+} influx. It activates enzymes that cause morphologic cellular alterations characteristic of necrosis and the consequent formation of a cystic cavity (Searle *et al.* 1982, Blagosklonny 2003, Renton *et al.* 2003). This notwithstanding, mild ischaemia has been shown to be able to induce apoptosis rather than necrosis (Chu *et al.* 2002, Blagosklonny 2003), and an abrupt impairment of oxygen supply does not occur in the periapical inflammatory lesion. However, no attempts have been made to investigate apoptotic epithelial cell death in the pathogenesis of ARC. A previous study enrolled this way of cell death in the formation of Rushton bodies', but asserted apoptosis as an aspect of necrosis (Pesce & Ferloni 2002). In this respect, such hyaline deposits were not seen in the present samples.

Even though the present results fail to explain cavity formation in ARC, and do not discard necrotic cell death in this regard, they do suggest the participation of apoptosis in the development of this lesion. Non-neoplastic proliferating cells respond to hypoxia and nutritional restrictions by activating genes able to induce cell cycle arrest and apoptosis (Chu *et al.* 2002, Renton *et al.* 2003). This action settles the proliferative activity by using the environmental conditions of the tissue (Blagosklonny 2003, Renton *et al.* 2003). In this regard, an attempt was made to investigate if lesions with atrophic lining had more apoptotic events than those with hyperplastic epithelium, since the former has been considered a less active lesion (Cury *et al.* 1998, Moreira *et al.* 2000). In fact, AI was higher in lesions with atrophic (mean = 0.170) rather than hyperplastic (mean = 0.098) epithelium, suggesting a role for apoptosis in the control of cell populations in radicular cysts. However, this difference was not

statistically significant. The use of a limited number of samples could have resulted in a type II statistical error, because of the low power of the test employed for the present analysis (not shown herein).

Regulation of apoptosis by the bcl-2 proteins family is known to be dependent on the relative level of inhibitory and inductive molecules (Adams & Cory 1998). Many feedback loops also play a significant role in the process (Blagosklonny 2003). In this meaning, the unexpected positive correlation between AI and bcl-2I may be the result of regulatory pathways acting on epithelial cells. Further research is suggested to explain this topic.

Conclusion

Apoptosis was always present in the epithelium of the ARC. Although it was more frequent in lesions with atrophic (quiescent) epithelium, there were no significant differences amongst the groups analysed. Other studies are needed to clarify the relative importance of this phenomenon in the development of radicular cysts, and to determine if such a finding could have an impact on the behaviour and the management of the disease.

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